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TITLE: A Gene Expression Profile of BRCAness That Predicts for Responsiveness to Platinum and PARP Inhibitors

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14. ABSTRACT Our results thus far support the hypothesis that the BRCAness profile is able to track diverse molecular mechanisms that cause defective homologous recombination (HR) and that it is associated with survival in patients with sporadic disease and clinical responsiveness to platinum. Specifically, application of the BRCAness profile in the TCGA EOC dataset can identify tumors that have defective HR due to overexpression of certain miRNAs, in the absence of known genetic and epigenetic abnormalities of the HR pathway. Furthermore, the HSP90 inhibitor 17-AAG enhances sensitivity of non-BRCA1/2 mutated ovarian cancer cells to DSB-inducing agents olaparib and carboplatin at very low, sublethal concentrations. Importantly, the mechanism for this synergistic effect seems to be increasing DNA damage via suppressing HR. Finally, high quality RNA from formalin fixed paraffin embedded ovarian cancer sections can be extracted and its detection and quantitation is highly concordant with that obtained from frozen tissue.					
15. SUBJECT TERMS ovarian cancer, homologous recombination, brcaness, PARP inhibitors, HSP90 inhibitors					
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1. INTRODUCTION:

Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

It is well established that hereditary ovarian cancer patients associated with germline BRCA-1 or BRCA-2 mutations exhibit two important clinical characteristics: i) heightened sensitivity to platinum analogues and PARP inhibitors (presumably due to an underlying defect in homologous recombination (HR)) and ii) improved survival compared to their sporadic counterparts. Importantly, certain sporadic tumors may also have abnormalities in HR (in the absence of germline BRCA mutations) and thus behave similarly to those with BRCA germline mutation. Such sporadic tumors are referred to as having a “BRCAness” phenotype that is characterized by heightened sensitivity to chemotherapy (platinum and PARP inhibitors) and improved overall survival. However, prospective identification of sporadic tumors with a BRCAness phenotype is challenging. This is because there are several molecular mechanisms that may underlie defective HR in a sporadic tumor in the absence of germline BRCA mutations. These mechanisms include epigenetic hypermethylation of the BRCA-1 gene promoter, somatic mutations of BRCA1 or BRCA2, loss of function mutations or epigenetic inactivation of other HR genes, and amplification of genes that inactivate BRCA gene function (e.g. EMSY which inactivates BRCA2). Given the heterogeneous mechanism(s) by which an ovarian cancer cell might harbor a BRCAness phenotype, we reasoned that a broad-based approach like gene expression profiling, which makes few assumptions about mechanism might have the highest chance of capturing sporadic tumors with a BRCAness phenotype. In this regard, we have developed a 60-gene expression profile that may identify tumors with a BRCAness phenotype. This profile designates tumors as BRCA-like (BL) or non-BRCA-like (NBL) corresponding to tumors predicted to have a BRCAness phenotype (BL tumors) or not (NBL tumors). In this grant we have proposed a series of studies to evaluate the ability of this profile to identify tumors with defects in homologous recombination, increased sensitivity to platinum and PARP inhibitors in vitro, clinical response to platinum and survival in patients with sporadic ovarian cancer and to identify compounds that can reverse PARP inhibitor resistance in vitro.

2. KEYWORDS:

Provide a brief list of keywords (limit to 20 words).

Epithelial Ovarian cancer, Homologous recombination, BRCAness, Gene expression profiling, PARP inhibitors, Platinum analogues, HSP90 inhibitors, Platinum Resistance, Nucleotide Excision Repair

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The major goal for Aim 1 was to determine whether the BRCAness gene expression profile is capable of prospectively identifying sporadic patients whose tumors exhibit defects in homologous recombination and increased sensitivity to platinum and PARP inhibitors in vitro.

The major goal for Aim 2 was to determine whether the BRCAness gene expression profile is associated with clinical response to platinum and survival in patients with sporadic ovarian cancer.

The major goal for Aim 3 was to evaluate whether the compounds identified by the Connectivity Map can reverse PARP resistance in vitro, and to investigate the mechanism for this effect.

The major goal for Aim 4 was to determine the reproducibility of the BRCAness profile when using the DASL mRNA assay in a cohort of FFPE ovarian cancer specimens with known clinical outcome and platinum responsiveness.

What was accomplished under these goals?

This is the final report of all the accomplishments for all four aims.

FOR AIM 1

We evaluated the ability of the BRCAness gene expression profile to identify defects in homologous recombination in several cellular systems.

The protein kinase ATM (ataxia-telangiectasia mutated) is a damage-response kinase that is responsible for the phosphorylation of Fanconi-Anemia (FA) proteins, which play an important role in DNA repair through homologous recombination (HR) (1). We have accessed gene expression (2) data of cells that were stably knocked down for ATM gene using retroviral vectors expressing the corresponding short hairpin RNA (shRNA). Gene expression data from control cells were also accessed. Cells that were stably knocked down for ATM gene had an impaired DNA damage response and an inactive HR DNA repair as opposed to control cells. Therefore cells knocked down for ATM and control cells afforded us the opportunity to assess whether the BRCAness profile could track cells with a defect in HR (in this case cells knocked down for ATM).

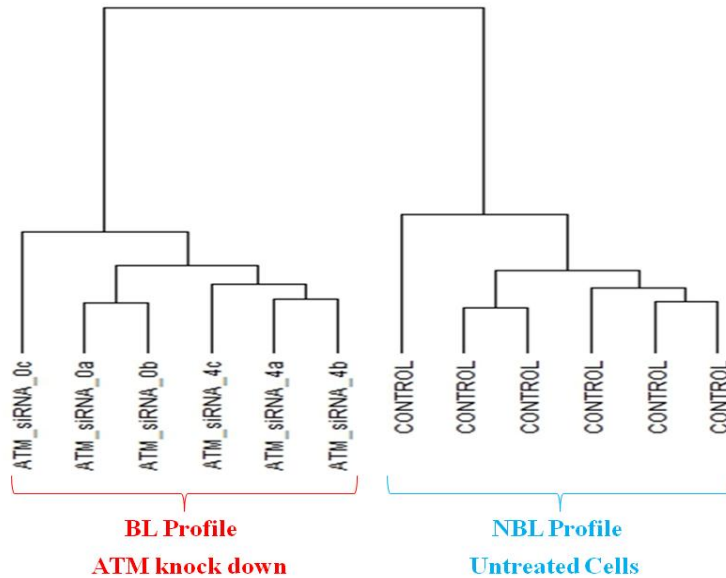


Figure 1. BRCAness profile distinguishes between ATM knock down and control cells

As shown in Figure 1 above, hierarchical clustering based on the expression pattern of the 60 genes of the BRCAness profile showed that the BRCAness profile could accurately distinguish between ATM knock down (HR deficient) cells and control (HR efficient) cells in 12 specimens. Specifically, six of six specimens with ATM knock down had the BL signature and six of six control specimens had the NBL signature (Fisher's exact two sided $p=0.002$).

We also evaluated the ability of the BRCAness profile to distinguish between cells that were knocked down for BRCA1 (via adenoviral BRCA1-RNAi) or control cells. Cells that were stably knocked down for BRCA1 gene had an inactive HR DNA repair as opposed to control cells and thus provided the opportunity to assess whether our profile could track a defect in HR in the absence of BRCA1 or BRCA2 mutations. Again, we accessed gene expression profiling data from BRCA1 knock down and control cells (3).

As shown in Figure 2 below, hierarchical clustering based on the expression pattern of the 60 genes of the BRCAness profile showed that the BRCAness profile could accurately distinguish between BRCA1 knock down (HR deficient) cells and control (HR efficient) cells in 6 specimens. Specifically, two of two specimens with BRCA1 knock down had the BL signature and four of four control specimens had the NBL signature (Fisher's exact two sided $p=0.067$).

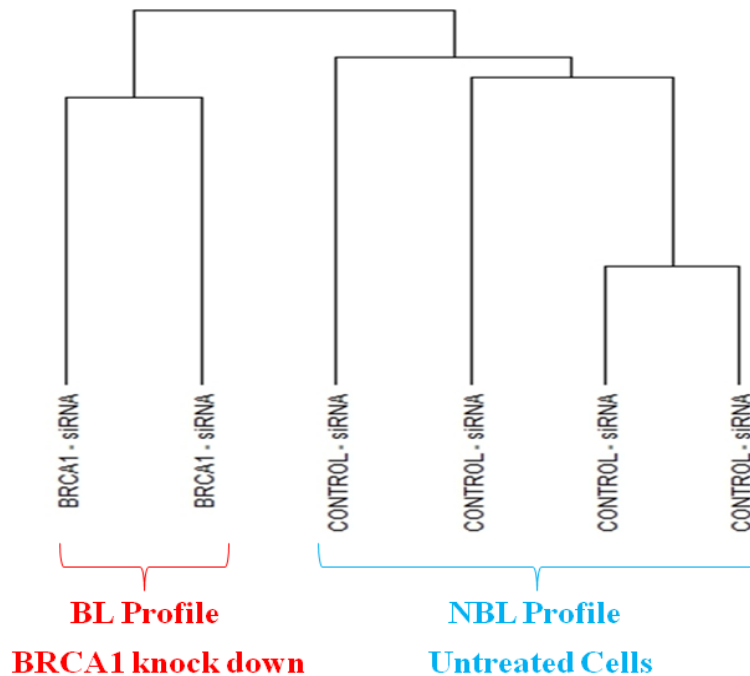


Figure 2. BRCAness profile distinguishes between BRCA1 knock down and control cells

The aforementioned findings (a+b) are very important because they show that our profile may be able to track defects in HR (i.e. ATM or BRCA1 knock down) in the absence of BRCA1 or BRCA2 mutations.

Furthermore, in order to evaluate whether the BRCAness gene expression profile was capable of identifying tumors with specific defects in homologous recombination we used the epithelial ovarian cancer (EOC) dataset from The Cancer Genome Atlas (TCGA) (4). The TCGA dataset is a unique tool for these studies as it includes gene expression data from tumors that are well characterized at a molecular level for defects in the homologous recombination pathway, including somatic BRCA1/2 mutations, epigenetic silencing of BRCA1, amplification or mutation of EMSY (an inhibitor of BRCA2), deficiency of PTEN (which has been identified to cause homologous recombination defects in human tumor cells) and mutations in Fanconi Anemia pathway, homologous recombination RAD and DNA damage sensing genes. The workflow of our study is summarized in Figure 3.

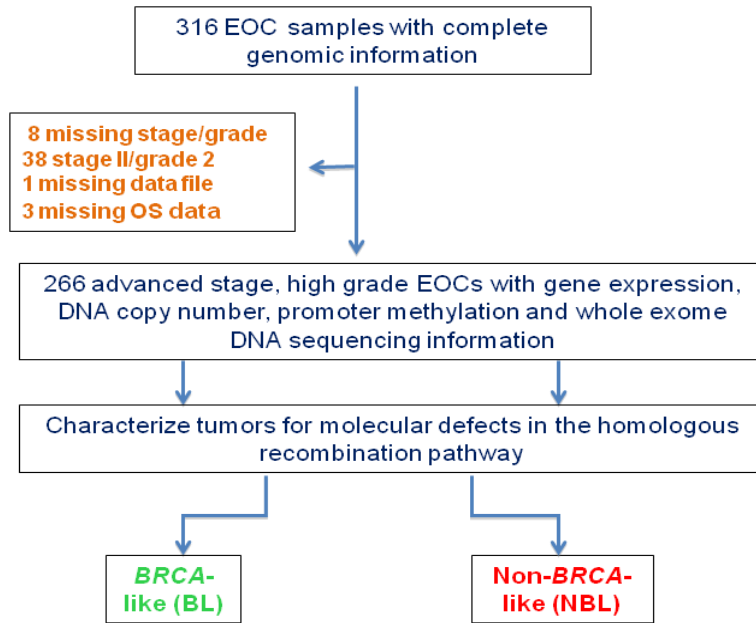


Figure 3. Study Workflow

Specifically, we accessed data from 316 serous EOCs for which there was available gene expression, DNA copy number, promoter methylation and whole-exome DNA sequencing information.

Of these 316 tumors, 50 tumors were excluded either because grade or stage were missing (n=8 tumors), or because they were early stage or grade 2 (n=38 tumors) or because there was missing microarray gene expression information (n=1 tumor) or overall survival data (n=3 tumors). Using the DNA copy number, promoter methylation and whole-exome DNA sequencing information, all remaining 266 tumors were characterized for known molecular defects in the homologous recombination pathway including germline or somatic BRCA1/2 mutations, epigenetic silencing of BRCA1, amplification or mutation of EMSY, deficiency of PTEN and mutations in Fanconi Anemia pathway, homologous recombination RAD and DNA damage sensing genes.

We then characterized these 266 tumors as having a BRCAness phenotype (BL profile) or not (NBL profile) based on our BRCAness signature and went on to evaluate whether BL tumors were enriched for specific defects in the HR pathway. The results are being summarized in Table 1 below.

BRCAness profile was associated with molecular events affecting BRCA1/2 function such as BRCA1/2 mutations, BRCA1 promoter hypermethylation and EMSY amplification. Specifically, BL patients were enriched in these events compared to NBL patients (47% vs 28% in NBL patients, two-sided Fisher's exact p=0.007, odds ratio 2.33, 95% C.I. 1.28-4.25). When including homozygous PTEN deletions, BL patients were again enriched in HR defects compared to NBL patients (56% vs 34% in NBL patients, two-sided Fisher's exact p=0.003, odds ratio 2.49, 95% C.I. 1.37-4.51). Similar results were obtained when including mutations or amplification of EMSY or other molecular events affecting the function of Fanconi Anemia (FA) genes (FANCA/B/C/D2/E/F/G/I/J/L/M, PALB2) or DNA damage response genes involved in HR

(ATM, ATR, CHEK1, CHEK2) or RAD genes (Table 1). When including all these HR defects, BL patients were enriched for these events compared to NBL patients (63% vs 47% in NBL patients, two-sided Fisher's exact $p=0.037$, odds ratio 1.92, 95% C.I. 1.05-3.51).

HR Defects	BL (%)	NBL (%)	p-value	Odds Ratio (95% CI)
BRCA 1/2 somatic (SM) and germline mutations plus BRCA1 promoter hypermethylation(HM)	47	28	0.007	2.33 (1.28-4.25)
Above + PTEN SM and homozygous deletion (HD)	56	34	0.003	2.49 (1.37-4.51)
Above + EMSY/c11orf30 amplification, HD, and SM	58	40	0.024	2.05 (1.14-3.72)
Above + RAD51C HM, Fanconi Anemia gene mutations, ATR/ATM mutations	63	47	0.037	1.92 (1.05-3.51)

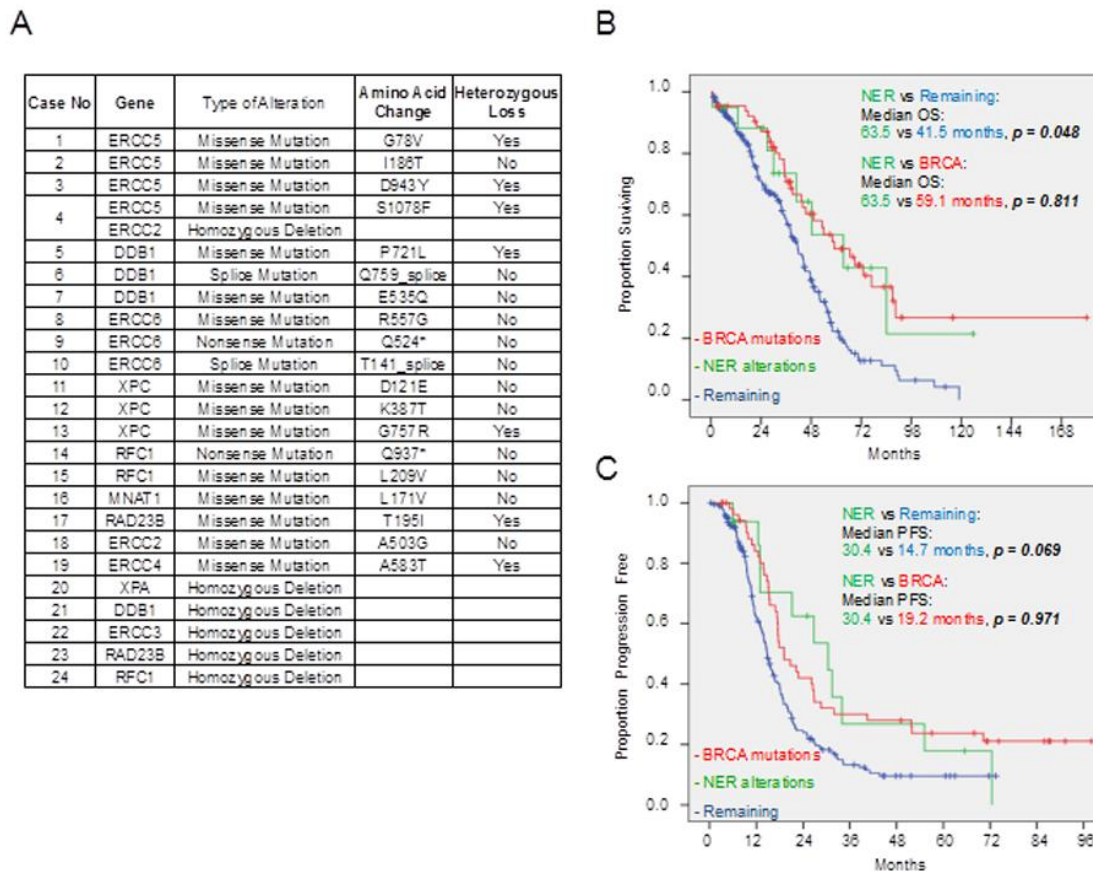
Table 1. Association of BRCAness profile with defects in HR pathway

These results were presented in the American Association of Cancer Research 2012 Annual Meeting in Chicago and show that the BRCAness profile may identify tumors who are enriched for diverse molecular aberrations of the homologous recombination pathway, far beyond the presence of germline mutations in the BRCA1 and BRCA2 genes. These findings are very important and represent a key validation step for our BRCAness profile because it is the first time that we show that it may be able to track various HR defects such as somatic BRCA1/2 mutations, epigenetic silencing of BRCA1, amplification or mutation of EMSY, deficiency of PTEN and mutations in Fanconi Anemia pathway, homologous recombination RAD and DNA damage sensing genes. As mentioned above, deficiency in homologous recombination is an important feature of BRCAness and may underlie improved responsiveness to drugs that induce double strand breaks such as platinum analogues and PARP inhibitors which are routinely repaired by the homologous recombination DNA repair pathway. Therefore, the ability of our profile to track diverse molecular mechanisms that cause defective HR represents an important validation step in terms of its ability to prospectively detect sporadic tumors with a BRCAness phenotype and provides a mechanistic basis of why this profile may correlate with responsiveness to chemotherapy in this disease.

Finally, we were able to define a unique subset of ovarian cancers which are not BRCAlike, i.e. not HR deficient and thus are resistant to PARPi but are sensitive to

platinum. Specifically, we curated the EOC TCGA dataset to assess potential inactivating events of the nucleotide excision repair (NER) pathway including mutations, homozygous deletions and promoter hypermethylation of NER genes. We found that a total of 24 (8%) of 316 EOCs harbored either NER mutations or homozygous deletions of NER genes (Fig. 4A). Specifically, we identified 19 cases with nonsynonymous or splice site NER gene mutations (all somatic) and 6 cases with homozygous deletions of NER genes among the 316 sequenced EOCs of the TCGA dataset. None of the NER genes were found to harbor promoter hypermethylation. All NER mutations were mutually exclusive, i.e. no individual tumor harbored mutations in more than one NER gene. Furthermore, NER mutations were mutually exclusive with homozygous deletions of the NER genes with the exception of one case that harbored both an ERCC5 mutation and homozygous deletion of ERCC2. Of the 19 cases with NER mutations, 7 (36.8%) were accompanied by heterozygous loss of the respective NER gene, indicating that in these cases both wild-type alleles had been lost. Importantly, patients with tumors with NER alterations exhibited higher median OS (63.5 vs 41.5 months respectively, log rank $p = 0.048$) and PFS (30.4 vs 14.7 months respectively, log rank $p = 0.069$) compared to patients with tumors without NER alterations and BRCA1/2 mutations (Fig. 4B and 4C). Furthermore, patients with tumors with NER alterations exhibited similar outcome (OS and PFS) with tumors harboring BRCA1 or BRCA2 mutations (Fig. 4B and 4C).

Figure 4.

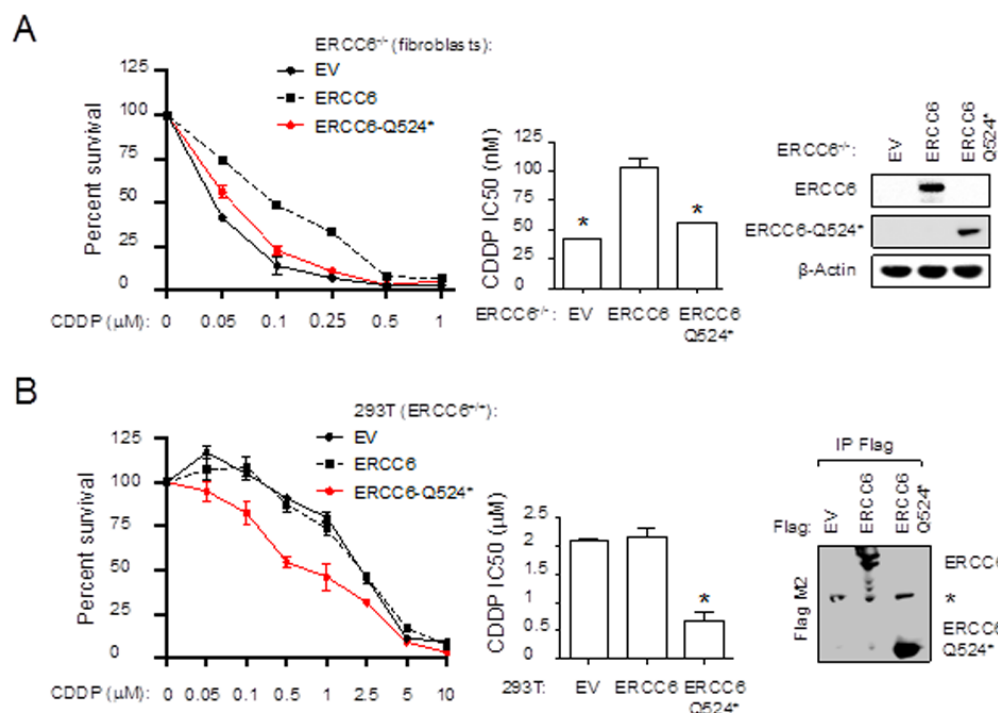


As a proof of principle that NER alterations are functionally associated with platinum sensitivity, we evaluated one NER mutation (ERCC6-Q524*) that was present in a patient with stage IV suboptimally-debulked high grade serous tumor that had complete response to first line platinum chemotherapy and remained in complete remission for 31.5 months after diagnosis.

In order to determine the functional significance of ERCC6-Q524* on platinum sensitivity, we evaluated whether this variant could rescue platinum sensitivity in an ERCC6-deficient cell line. An ERCC6-deficient immortalized fibroblast cell line (GM16095) was complemented with either wild-type ERCC6 or the mutant ERCC6-Q524*. Expression of wild-type ERCC6 rescued cisplatin sensitivity of ERCC6-deficient cells while complementation with mutant ERCC6-Q524* did not impact cisplatin sensitivity (Fig. 5A). In order to confirm that ERCC6 loss alone is solely sufficient to induce cisplatin sensitivity, we assessed cisplatin cytotoxicity following siRNA knockdown of ERCC6. ERCC6 depletion significantly increased platinum sensitivity, comparable to BRCA2 loss, a major mediator of DNA crosslink repair.

Given that ERCC6-Q524* was a somatic mutation and not associated with heterozygous loss, we evaluated whether this mutation may exert a dominant negative effect. We postulated that ERCC6-Q524* may interfere with the function of the wild-type allele and hence increase sensitivity to cisplatin. Indeed, introduction of the ERCC6-Q524* variant in ERCC6 wild-type 293T cells dramatically increased cisplatin sensitivity compared to cells transfected either with wild-type ERCC6 or control empty vector suggesting that this mutation sensitizes cells to cisplatin by a dominant negative mechanism (Fig. 5B).

Figure 5.



We evaluated the association of this NER mutation with sensitivity to the PARPi rucaparib. Unlike in the case of cisplatin, expression of wild-type or mutant ERCC6 did not affect PARPi sensitivity of ERCC6-deficient fibroblasts. Furthermore, since defective HR is a critical mediator of platinum and PARPi sensitivity in EOC, we evaluated whether deficiency in ERCC6 affected HR in vitro. Inhibition of ERCC6 did not affect HR efficiency in vitro, as measured by direct-repeat GFP recombination (DR-GFP) assay and by IR-induced RAD51 foci formation, a surrogate for HR efficiency. Together, these results indicated that functional loss of ERCC6 does not impair HR efficiency nor alters sensitivity to PARPi or other double strand break-inducing agents such as doxorubicin. These findings, which were published in Cancer Research (5) showed for the first time that NER pathway alterations (mutations and homozygous deletions) occur in EOC and that these alterations are associated with a phenotype of clinical platinum sensitivity that is similar to that of BRCA1/2-mutated tumors characterized by improved overall and progression free survival. These findings suggest that NER alterations may have a previously unrecognized role as biomarkers for selection of patients for participation in PARPi trials as well as for deciding therapy after development of PARPi resistance.

FOR AIM 2

In order to evaluate whether the BRCAness gene expression profile is associated with clinical response to platinum and survival in patients with sporadic ovarian cancer we used a patient cohort that included 70 patients treated at Beth Israel Deaconess Medical Center, Memorial Sloan-Kettering Medical Center, and Cedars-Sinai Medical Center. All these patients had undergone exploratory laparotomy for diagnosis, staging, and debulking followed by first-line platinum-based chemotherapy. Standard post-chemotherapy surveillance included serial physical examination, serum CA-125 level, and computed tomography scanning as clinically indicated. This 70-patient cohort included two 35-patient cohorts as follows: The first cohort included tumor samples from 35 patients with invasive EOC who underwent sequencing for germline mutation (by using DNA obtained from peripheral-blood leukocytes) and did not harbor germline BRCA1 or BRCA2 mutations. The second cohort included 35 patients who did not undergo genetic testing but who were enriched for sporadic disease on the basis of the following characteristics: no family history of ovarian cancer, no family history of breast cancer younger than 50 years of age, no family history of more than one breast cancer at any age, and not of Ashkenazi Jewish ethnicity.

Total RNA was isolated from patient tumor samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA synthesis and hybridization on oligonucleotide microarrays (U133 Plus 2.0 Array GeneChip, Affymetrix, Inc., Santa Clara, CA) containing approximately 54,700 transcripts were carried out using standard protocols. Microarray experiments were performed at the Dana Farber Cancer Institute Microarray Core Facility (<http://chip.dfci.harvard.edu/>). Raw data were processed using Robust Multi-Array (RMA) analysis. All raw microarray data have been deposited in GEO (Gene Expression Omnibus - GSE19829). These data have been published in Journal of Clinical Oncology (6).

Overall, 20 (29%) of the 70-patient cohort demonstrated the BL profile (eight of 35 in the sequenced group, and 12 of 35 in the non-sequenced group). As listed in Table 2 below, there were no differences in age, stage, grade, histology, or debulking status between the BL and the NBL signature groups. The ability to achieve a clinical remission for the BL and NBL groups was 90% compared with 74%, although this did not reach statistical significance (two-sided Fisher's exact $P=0.2$).

Characteristic		NBL Profile (n=50)		BL Profile (n=20)		p value
		No.	%	No.	%	
Age	Median (range)	61 (39-89)		59.25 (44-80)		0.55
Grade	1-2	9	18	1	5	0.477
	3	41	82	19	95	
Histology	Serous	46	92	19	95	0.412
	Clear Cell	1	2	1	5	
	Endometrioid	3	6	0	0	
Stage	2	3	6	0	0	0.479
	3	41	82	16	80	
	4	6	12	4	20	
Debulking Status	Optimal	33	67.3	16	80	0.386
	Suboptimal	16	32.7	4	20	
Achievement of CR after first- line therapy		74%		90%		0.2

Table 2. Association of BRCAness profile with clinical characteristics and remission status after first line therapy

For the entire 70-patient cohort, the BRCAness profile was capable of discriminating between long and short median disease free survival (DFS); the patients with BL and NBL profiles had median DFS times of 34 months and 15 months, respectively (log-rank $P = 0.013$; Figure 6). In addition, the percentages of patients who were disease free at 4 months for the BL and NBL groups were 90% and 64% ($P =$

0.04), respectively; at 6 months, percentages were 85% and 60%, respectively ($P = 0.053$); and at 18 months, percentages were 65% and 29%, respectively ($P=0.007$).

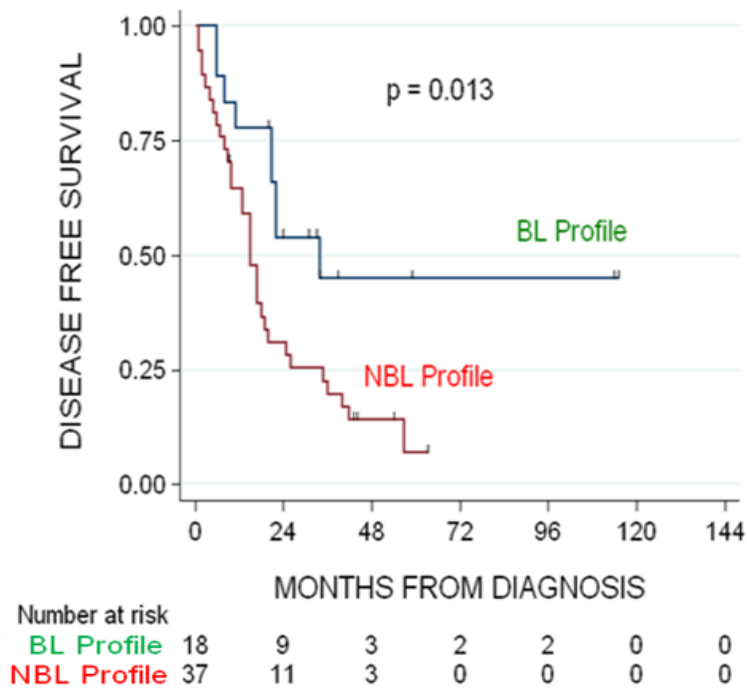


Figure 6. Association of BRCAness profile with DFS. Median DFS for BL and NBL patients was 34 and 15 months, respectively ($P = 0.013$).

Furthermore, the BRCAness profile distinguished between long and short median overall survival (OS), as the patients in the BL and NBL groups had median OS times of 72 and 41 months, respectively (log-rank $P = 0.006$; Figure 6). Similar findings were observed when applying the profile separately to the group of 35 sequenced patients who had undergone germline mutation testing and who were found to have wild-type BRCA1 and BRCA2 genes or to the group of the 35 non-sequenced patients enriched for sporadic disease on the basis of clinical characteristics, as previously described.

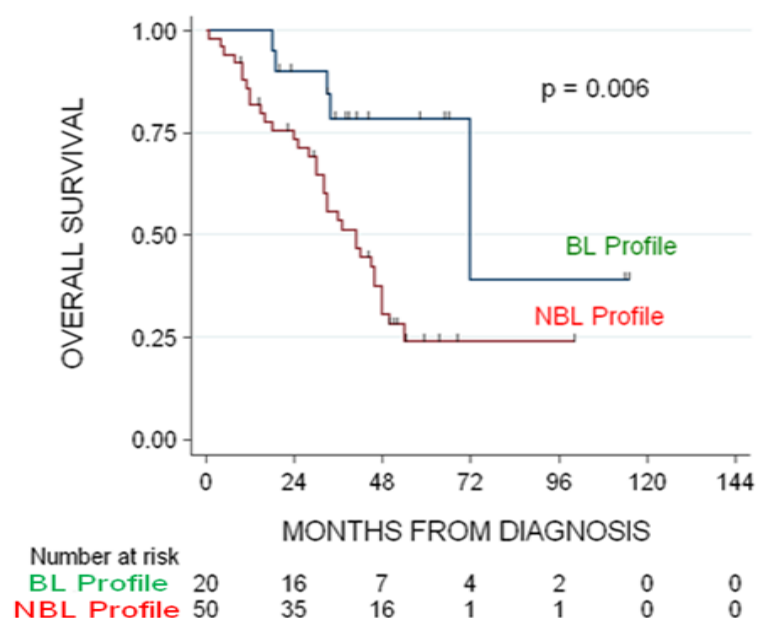


Figure 7. Association of BRCAness profile with OS. Median OS for BL and NBL patients was 72 and 41 months, respectively ($P = 0.006$).

In univariate analysis, the hazard ratio for recurrence (NBL v BL group) was 2.47 ($P=0.018$; 95% CI, 1.17 to 5.2), and the hazard ratio for death (NBL v BL group) was 3.29 ($P=0.009$; 95% CI, 1.34 to 8.09). Multivariate analysis, which included the BRCAness profile, age, stage, grade, histology, and debulking status, demonstrated that the profile maintained an independent association with DFS and OS (Table 3). The hazard ratio for recurrence (NBL v BL group) was 2.65 ($P=0.016$; 95% CI, 1.2 to 5.86), and the hazard ratio for death (NBL v BL group) was 3.39 ($P=0.009$; 95% CI, 1.35 to 8.5).

Factor	Univariate P Value		Multivariate P Value	
	DFS	OS	DFS	OS
Age	0.44	0.35	0.96	0.8
Grade	0.9	0.34	0.87	0.32
Histology	0.97	0.61	0.9	0.96
Stage	0.43	0.54	0.4	0.61
Debulking Status	0.39	0.05 (HR=1.84)_a	0.53	0.67
NBL/BL profile	0.018 (HR=2.47)	0.009 (HR=3.29)	0.016 (HR=2.65)	0.009 (HR=3.39)

Table 3. Predictive value of BRCAness profile adjusted for Grade, Age, Histology, Stage and Debulking

In conclusion, these data support our hypothesis that the BRCAness profile is associated with survival as well as with clinical response to platinum among patients with sporadic disease. Sporadic tumors with the BRCAlike (BL) profile were associated with increased overall survival as well as platinum sensitivity as evaluated using several metrics of platinum response including achievement of complete response after platinum chemotherapy, disease free survival and percentage of patients who are disease free at 4 months, 6 and 18 months. Of note, multivariate analysis which included the BRCAness profile, age, stage, grade, histology, and debulking status, demonstrated that the profile maintained an independent association with DFS and OS.

FOR AIM 3

By applying the BRCAness profile to the Connectivity Map, we identified that HSP90 inhibitors may be functionally associated with induction of defective HR and reversion of HR proficient to HR-deficient tumors. Furthermore, analysis of publicly available gene expression data showed that exposure of HR-proficient cancer cell lines to the HSP90 inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin) statistically significantly downregulated HR ($p<0.005$), ATM ($p=0.015$) and Fanconi Anemia ($p<0.005$) pathways, and downregulated the expression levels of several genes of these pathways. Importantly, in HR-proficient ovarian cancer cells, sublethal doses of HSP90i 17-AAG suppressed HR as assessed using the RAD51 foci formation after ionizing radiation (IR) assay and using the Direct Repeat-GFP (DR-GFP) reporter assay (Figure 8).

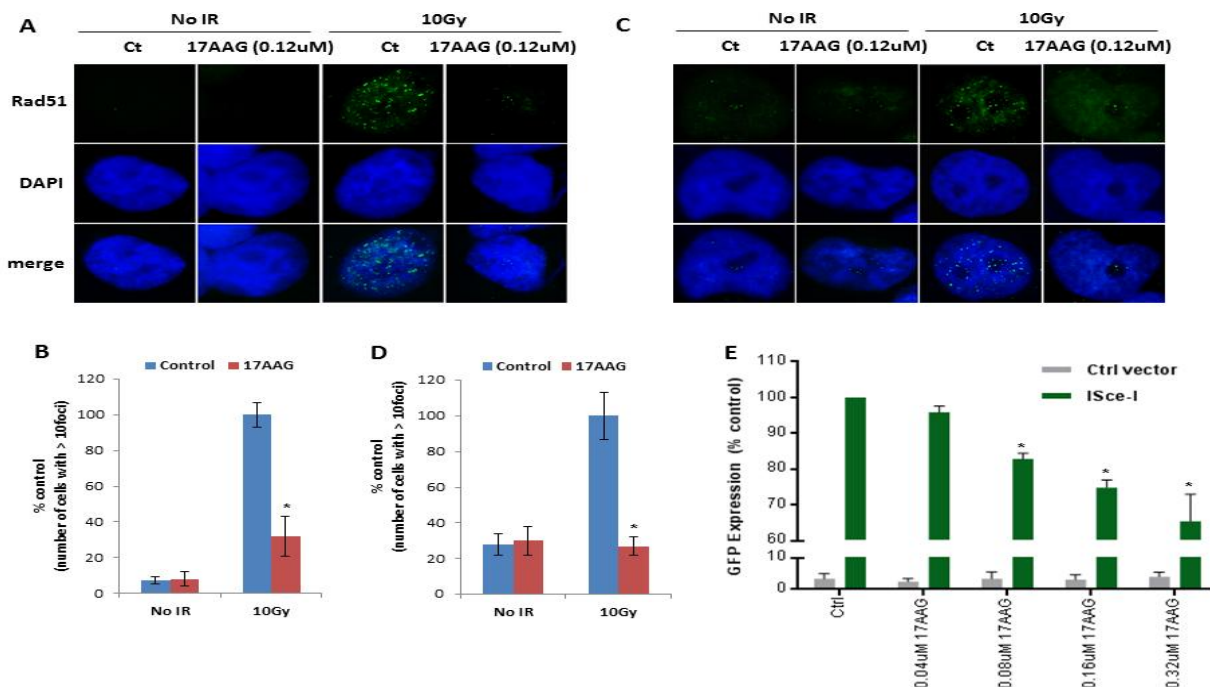


Figure 8. 17-AAG downregulates HR-mediated DSB repair. (A-D) Analysis of HR-mediated repair by RAD51 focus formation. 36M2 cells (A and B) or SKOV3 cells (C and D) were treated with 17-AAG or vehicle control for 24 hrs, stained for RAD51 (green) and DAPI (blue) 6 h after exposure to IR. (E) Measurement of HR-mediated repair of an I-SceI induced site specific DSB. Cells carrying a single copy of the recombination substrate (DR-GFP) were treated with indicated concentrations of 17-AAG or vehicle control for 24 hrs before transfection with I-SceI or control vector.

Additionally, sublethal doses of 17-AAG downregulated protein levels of BRCA1 and/or RAD51, and induced significantly more γ H2AX activation in combination with olaparib compared to olaparib alone in HR proficient ovarian cancer cells (Figure 9).

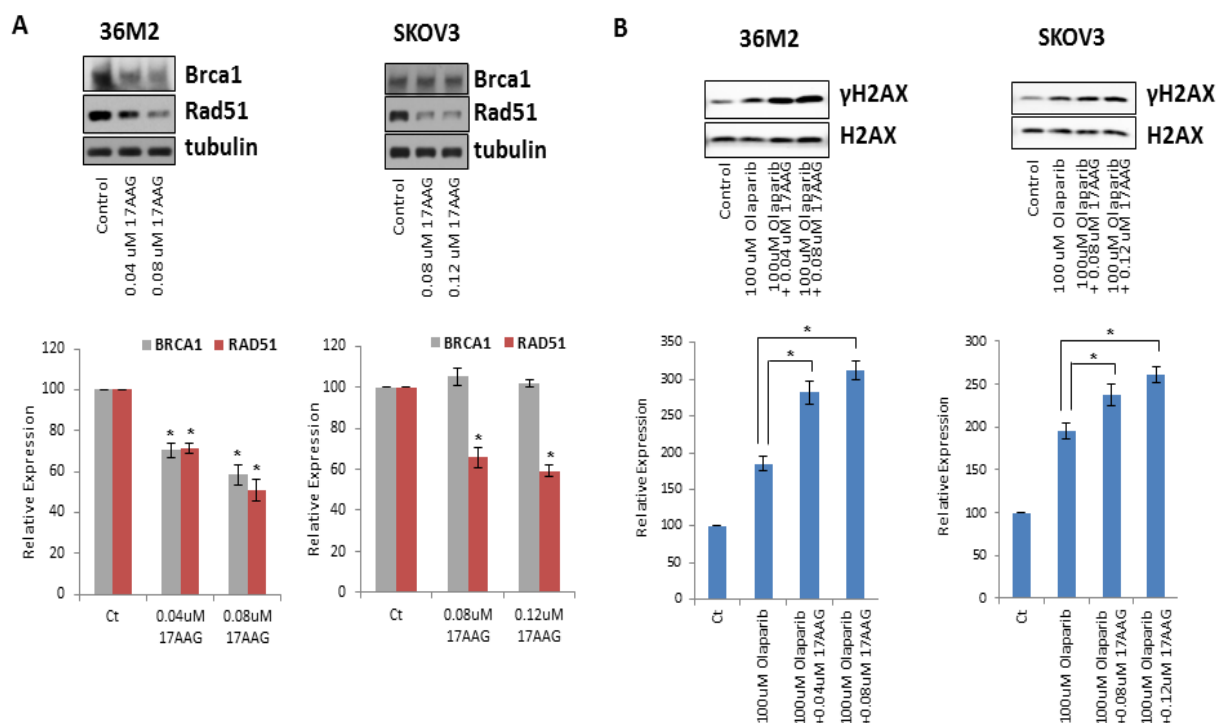


Figure 9. 17-AAG downregulates BRCA1 and/or RAD51. (A) Indicated cells were treated with 17-AAG or vehicle control for 24 hrs and washed off before subjected to immunoblotting in another 24 hrs. Cell lysates were analyzed by immunoblot for BRCA1 or RAD51 (B) γ -H2AX accumulation after treatment with olaparib \pm 17-AAG. Indicated cells were treated with olaparib \pm 17-AAG for 24 hrs before evaluation of γ -H2AX by immunoblotting.

Importantly, sublethal concentrations of 17-AAG sensitized HR proficient ovarian cancer lines to both olaparib and carboplatin (Figure 10).

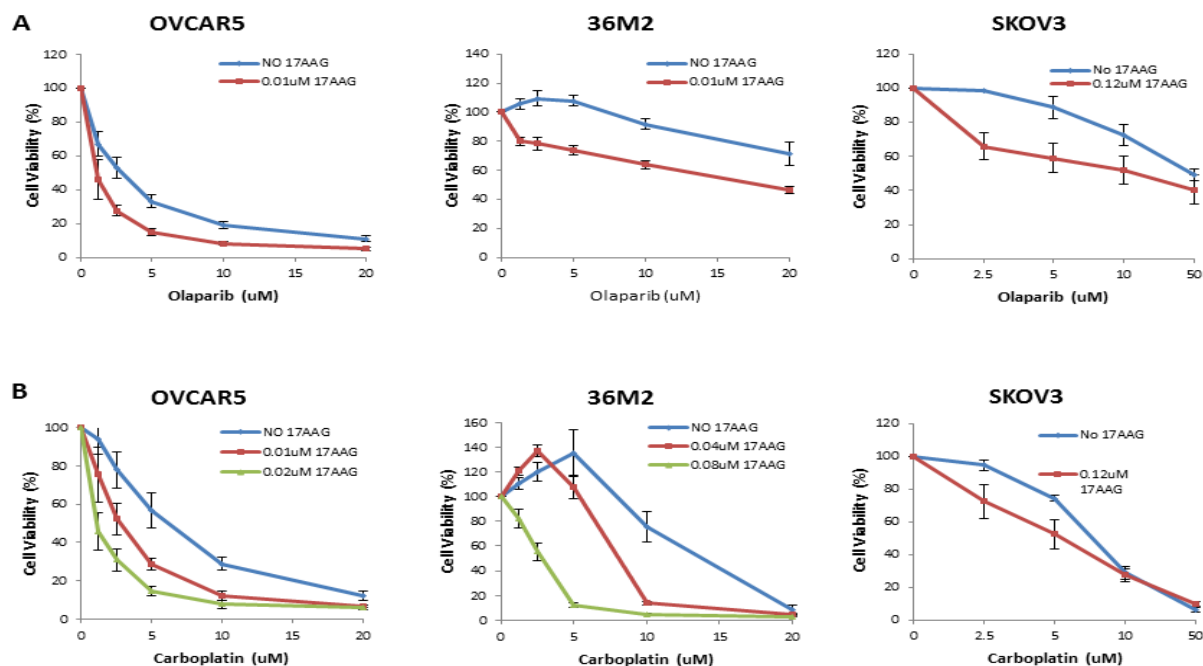


Figure 10. 17-AAG sensitizes HR-proficient cells to olaparib and carboplatin. (A and B) Luminescence-based viability assay in HR-proficient cells with olaparib or carboplatin. Cells were plated onto a 96-well plate at 1000 cells/well density and treated with indicated concentrations of PARP inhibitor, olaparib (A) or platinum drug, carboplatin (B) on the following day. Viability was tested by using CellTiter Glo (Promega) in 5 days. Curves were generated from 3 independent experiments.

Apart from HR proficient cell lines, we also evaluated the effect of 17-AAG in HR deficient ovarian cancer cells, specifically the OVCAR8 cell line which harbors almost undetectable levels of BRCA1 protein (Figure 11A) and very low levels of BRCA1 transcript (Figure 11B) compared to 36M2 ovarian cancer line. As shown in Figure 11C, sublethal concentrations of 17-AAG did not sensitize OVCAR8 cells to olaparib or carboplatin. This finding strongly suggests that the 17-AAG induced sensitization to olaparib and carboplatin is related to suppression of HR and not due to another (HR-independent) mechanism. Although our data suggest that 17-AAG suppresses HR and sensitizes to olaparib and carboplatin in vitro, we would like to recognize that 17-AAG-induced sensitization to platinum and PARP-inhibitors need to be further confirmed using in vivo models.

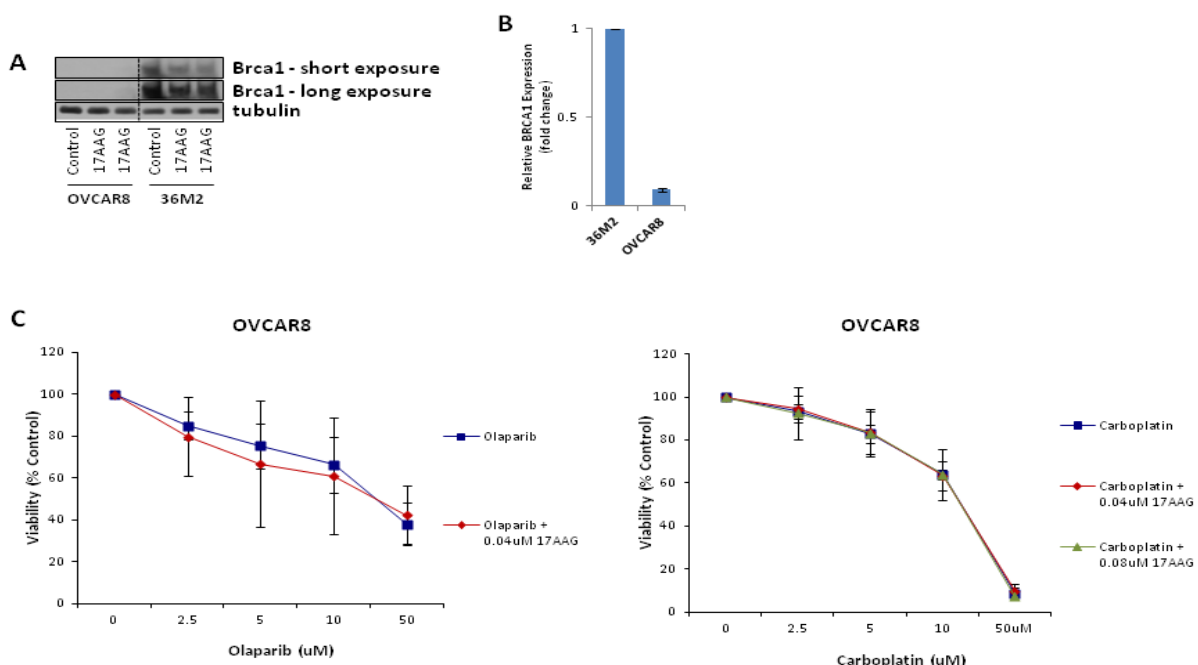


Figure 11. 17-AAG does not sensitize HR-deficient cells to olaparib or carboplatin. (A and B) Validation of undetectable levels of BRCA1 expression in OVCAR8 cells. OVCAR8 cells were analyzed for BRCA1 expression by immunoblotting (A) and qRT-PCR (B) compared to BRCA1-proficient 36M2 cells. (C) Viability assay in HR-deficient cells with PARP inhibitor or platinum drug. Viability assay was done in the same way as in Figure 3.

These results which have been published in 2014 (7), suggest that sublethal concentrations of the HSP90i 17-AAG suppress HR and enhance sensitivity of HR proficient ovarian cancer cells to platinum and PARPis.

Finally, in addition to the in vitro data, we have now performed experiments to assess whether HSP90 inhibitors identified via the Connectivity Map can revert PARPi resistance in vivo. Specifically, we used a PARPi resistant patient xenograft model and we performed efficacy studies. Strikingly, this model was PARPi resistant and was also resistant to combinations of olaparib and the PI3K inhibitors BKM120 or BYL719. Olaparib was dosed at 100mg/kg po daily x 4 weeks, AT13387 (HSP90i) was administered at 45mg/kg po for 2 days (D1,D2) on / 5 days off x 4 weeks (i.e. Days 1, 2, 8, 9, 15, 16, 22, 23). As shown in Figure 12, the combination of AT13387 and olaparib induced inhibition of tumor growth as opposed to vehicle control, olaparib alone and AT13387 alone.

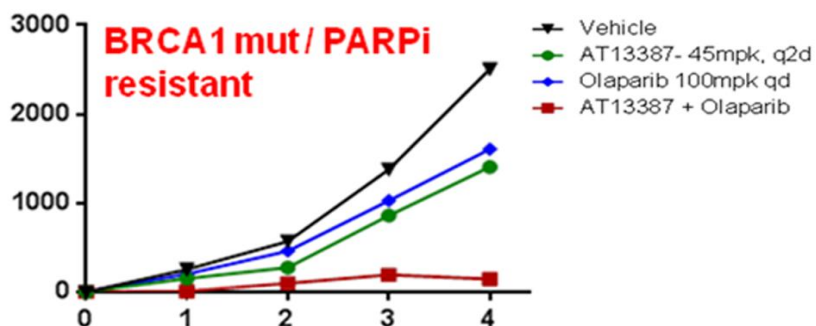


Figure 12. Synergism between PARP-inhibition and HSP90-inhibition in a PARPi-resistant patient-derived xenograft model.

Together, all these results provide a preclinical rationale for using a combination of 17-AAG and olaparib and/ or carboplatin in EOCs that are HR proficient either at baseline or at the time of development of platinum or PARPi resistance which was a major goal of our project.

FOR AIM 4

In addition to the 70 patients identified and discussed above in Aim 2, we have identified additional 68 patients at Dana-Farber Cancer Institute (DFCI), for a total of 138 patients. This cohort of patients is characterized as BRCAlike and nonBRCAlike. Of the last 68 patients, 53 have been molecularly characterized. Specifically, there was a BRCA1/2-mutated group comprised of 37 HGSOEs (29 with BRCA1 and 8 with BRCA2 mutations) with BRCA1/2 germline mutations identified by genetic testing (Figure 4). There was also a HR-proficient group (i.e. group of tumors without HR alterations) comprised of 16 ovarian cancers which were identified in a two-step process (Figure 4). First, Next Generation Sequencing (NGS) was performed to exclude tumors with mutations in HR genes; this analysis identified 17 such tumors (Figure 4).

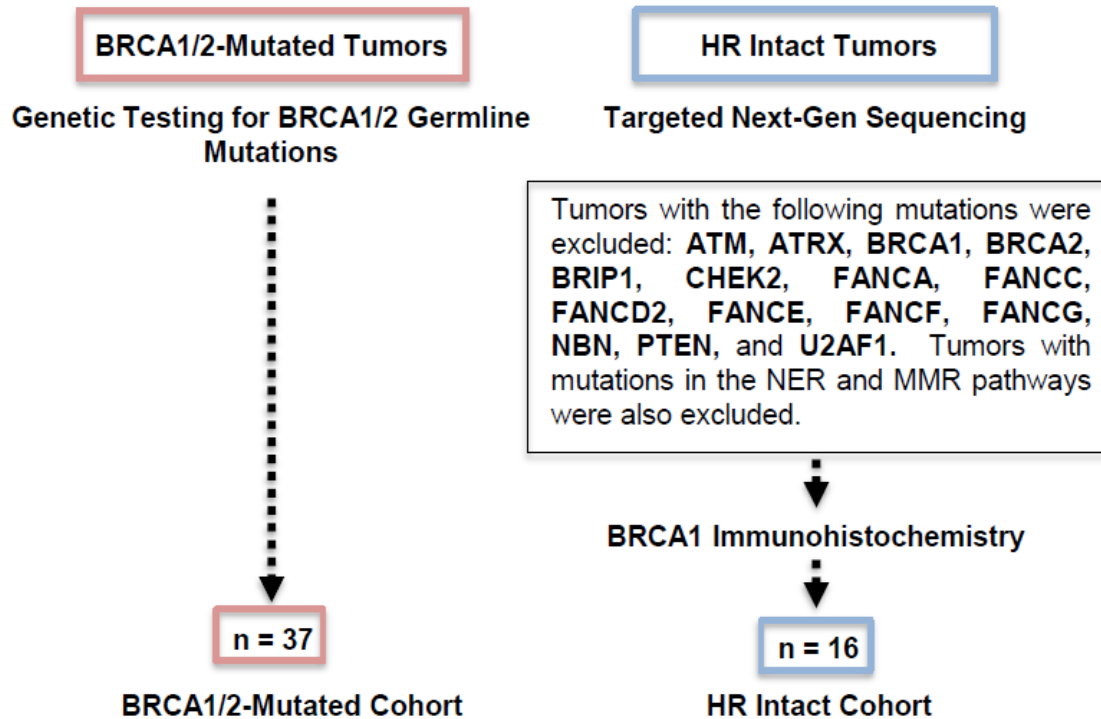
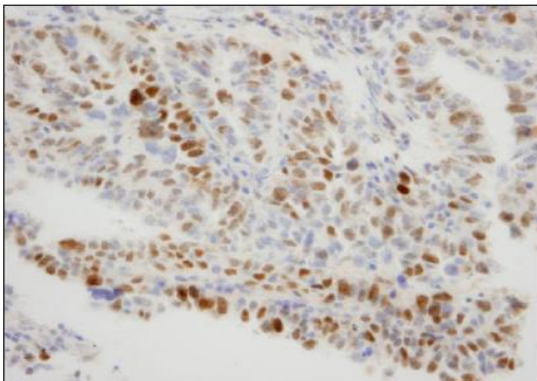


Figure 13. Additional 53 patients identified at DFCI.

These 17 tumors were subsequently evaluated for BRCA1 expression by immunohistochemistry to exclude the possibility of BRCA1 promoter hypermethylation that would lead to absent BRCA1 expression. As a result of this testing, 1 tumor was found to have staining in less than 5% of tumor cells with the presence of a strong internal control (Figure 14), which was then excluded from the HR-proficient(HR intact) group. Interestingly, review of the NGS data for this case demonstrated that this tumor had a single copy deletion of the BRCA1 gene, suggesting that BRCA1 loss was likely due to single copy deletion of BRCA1 and epigenetic silencing of the complementary allele. Ultimately, the HR proficient group consisted of 16 tumors without mutations in HR pathway genes and without BRCA1 loss by IHC (Figure 13).

A.



B.

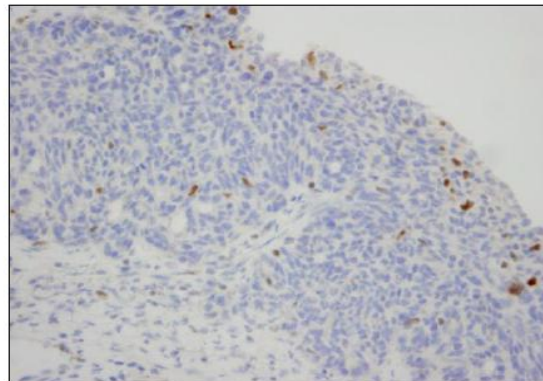


Figure 14. Results of BRCA1 immunohistochemistry. (A) Positive BRCA1 IHC in a representative case. BRCA1 expression was positive by IHC in 16 of the 17 tumors without HR alterations identified by NGS. (B) BRCA1 IHC was negative in one tumor that did not harbor HR alterations by NGS. Focal BRCA1 positivity was present in lymphocytes.

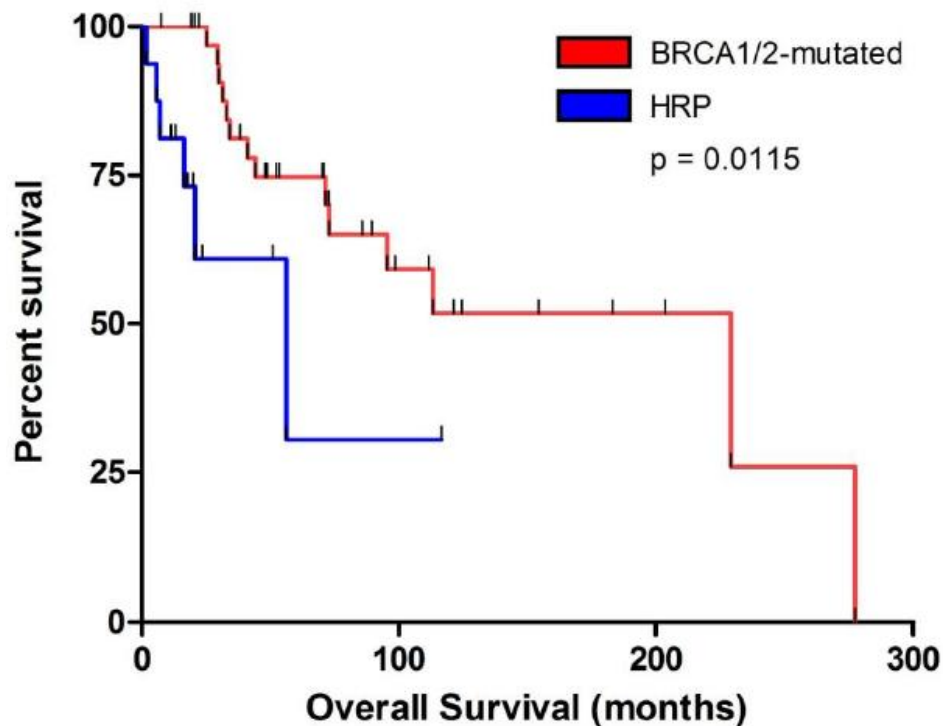


Figure 15. Overall survival of patients with BRCA1/2-mutated (red) versus HR intact (HRP, blue) tumors.

Importantly, as shown in Figure 15 above, BRCA1/2-mutated tumors exhibited improved OS compared to HR-proficient HGSOCS ($p=0.012$).

We are in the process of finalizing the characterization of the remaining 15 patients which will complete the full characterization of the 138 patients.

What opportunities for training and professional development did the project provide?

When I received this award, I was instructor of Medicine at Harvard Medical School. During this award, I was promoted to Assistant Professor of Medicine on 2011 and at this point, my promotion to Associate Professor of Medicine is in Progress. Furthermore, I moved to Dana-Farber Cancer Institute Gynecologic Oncology Program. During this award, I have also been a member of prestigious national committees including being a member of the GOG/NRG Experimental Medicine Committee, serving as a member of the Ovarian Task Force of the NCI Gynecologic Cancer Steering Committee and also served as Co-chair of the ASCO Expert Panel for development of clinical guidelines on Germline and Somatic Tumor Testing in Gynecologic Cancers.

How were the results disseminated to communities of interest?

The results of the work during the award has been published and presented in national meetings as discussed in detail below. Furthermore, microarray data with the generated RNA expression data of the sporadic patients has been deposited in the publicly available Gene Expression Omnibus (GEO), <http://www.ncbi.nlm.nih.gov/geo/>, Accession number GSE19829.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

Nothing to Report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

- BRCAness profile is able to track diverse molecular mechanisms that cause defective homologous recombination (HR) including somatic BRCA1/2 mutations, epigenetic silencing of BRCA1, amplification or mutation of EMSY, deficiency of PTEN and mutations in Fanconi Anemia pathway, homologous recombination RAD and DNA damage sensing genes
- BRCAness profile is able to track also in vitro other molecular mechanisms that cause defective homologous recombination (HR) including knockdown of ATM and BRCA1
- BRCAness profile is associated with survival in patients with sporadic disease

- BRCAness profile is associated with clinical sensitivity to platinum as evaluated using various metrics of platinum response in sporadic patients
- We have discovered a novel mechanism of discordance between platinum and PARPi sensitivity in ovarian cancer that involved NER alteration. Our findings suggest that NER alterations may have a previously unrecognized role as biomarkers for selection of patients for participation in PARPi trials as well as for deciding therapy after development of PARPi resistance.
- HSP90 inhibitor 17-AAG can enhance sensitivity of ovarian cancer cells to PARP inhibitor olaparib and to carboplatin
- The mechanism of this previously unknown, off-target effect of 17-AAG seems to be due to increasing DNA double strand breaks. This is an important finding which supports a trial of 17-AAG with platinum or olaparib in ovarian cancer
- The promise of PARP inhibitors in the management of ovarian cancer is tempered by the fact that HR-proficient cancers do not respond well to these agents, suggesting that approximately 50% of ovarian cancer patients (i.e. those without HR alterations) do not benefit from this novel class of drugs. Combination of PARPis with agents that inhibit HR may represent an effective strategy to sensitize HR proficient tumors to PARPis and thus potentially expand use of these agents beyond patients with HR deficient EOCs. Our in vivo findings that HSP90is revert PARPi resistance in xenografts provide the preclinical rationale for using a combination of 17-AAG and olaparib and/ or carboplatin in ovarian cancers that are HR proficient either at baseline or at the time of development of platinum or PARPi resistance. This can have a significant impact on patients who develop resistance to PARP-inhibitors or platinum analogues as the combination of 17-AAG/PARP-inhibitors or 17-AAG/carboplatin may effectively overcome this problem.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS:

Journal publications.

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Books or other non-periodical, one-time publications.

1. Klopp AH, Eifel PJ, Berek JS, Konstantinopoulos PA. Chapter 72: Cancer of the Cervix, Vagina and Vulva (2015). In: DeVita VT, Lawrence TS, and Rosenberg S. Cancer: Principles & Practice of Oncology. 10th edition. Philadelphia: Wolters Kluwer Health/Lippincott Williams and Wilkins. p. 1013-47.
2. Battelli C, Konstantinopoulos PA. Chapter 60: Gynecologic Cancers (2013) in Rose MG, DeVita VT, Lawrence TS, Rosenberg S. Oncology for the Primary Care Clinician Textbook.
3. Liu JF and Konstantinopoulos PA. Chapter: Homologous recombination and BRCA genes in ovarian cancer: clinical perspective of novel therapeutics. In: Birrer M, Ceppi L. Translational Advances in Gynecologic Cancers, Elsevier, in press.

Other publications, conference papers, and presentations.

Abstracts in National Meetings:

- 1) Panagiotis A. Konstantinopoulos, Vicky Makker, William T. Barry, Joyce F. Liu, Neil S. Horowitz, Michael J Birrer, L. Austin Doyle, Suzanne Berlin, Christin Whalen, Paul Van Hummelen, Robert Coleman, Carol Aghajanian, Gordon B. Mills, Ursula A. Matulonis, Shannon Neville Westin, Andrea P. Myers. Phase II, single stage, cohort expansion study of MK-2206 in recurrent endometrial serous cancer. (2014) Abstract 5515, 2014 ASCO Meeting, Chicago, IL.
- 2) Carolyn N. Krasner, Michael J. Birrer, Suzanne T. Berlin, Neil S. Horowitz, Mary K. Buss, Scott Eliasof, Edward Graeme Garmey, Meliessa G. Hennessey, Panagiotis Konstantinopoulos, Ursula Matulonis. Phase II clinical trial evaluating CRLX101 in recurrent ovarian, tubal, and peritoneal cancer. (2014) Abstract 5581, 2014 ASCO Meeting, Chicago, IL.
- 3) Raphael Ceccaldi, Kevin O'Connor, Kent Mouw, Ursula Matulonis, Alan D'Andrea, Panagiotis A. Konstantinopoulos. Nucleotide Excision Repair (NER) alterations confer sensitivity to platinum but not PARP inhibitors in epithelial ovarian cancer. 10th Biennial Ovarian Cancer Research Symposium, 2014, Seattle, WA.
- 4) Ursula Matulonis, Alexi A. Wright, Siobhan A Collins, Susana M. Campos, Panagiotis Konstantinopoulos, Kimberley MacNeill, Stephanie Morrissey, Victoria Patterson, Christin Whalen, Joyce Liu. A single arm, single stage phase II trial of trametinib (GSK1120212) and GSK2141795 in persistent or recurrent cervical cancer. (2015) Abstract TPS5618, 2015 ASCO Meeting, Chicago IL.
- 5) Carolyn N. Krasner, Michael J. Birrer, Suzanne T. Berlin, Mary K. Buss, Scott Eliasof, Edward Graeme Garmey, Meliessa Hennessey, Neil S. Horowitz, Panagiotis Konstantinopoulos, Ursula A Matulonis. Targeting VEGFRi resistance through HIF-1 α suppression: Phase II clinical trial evaluating CRLX101 as monotherapy and in combination with bevacizumab in recurrent platinum

- resistant ovarian cancer. (2015) Abstract TPS5614, 2015 ASCO Meeting, Chicago IL.
- 6) Richard T. Penson, Suzanne T. Berlin, Panagiotis Konstantinopoulos, Carolyn N. Krasner, Ursula Matulonis, Allison Ambrosio, Daphne Suzin, Siobhan A Collins, Michael J. Birrer, Yael Chava Cohen. A phase I/II trial of multiple dose VB-111 and weekly paclitaxel in recurrent platinum-resistant Müllerian cancer. (2015) Abstract 5542, 2015 ASCO Meeting, Chicago IL.
 - 7) Panagiotis Konstantinopoulos, Sangeetha Palakurthi, Qing Zeng, Shan Zhou, Joyce F. Liu, Elena Ivanova, Cloud Paweletz, Naveen Kommajosyula, Alan D. D'Andrea, Geoffrey Shapiro, Ursula A. Matulonis. In vivo synergism between PARP-inhibitor olaparib and HSP90-inhibitor AT13387 in high grade serous ovarian cancer patient derived xenografts. (2016) e17045, 2016 ASCO Meeting, Chicago IL.
 - 8) Panagiotis Konstantinopoulos, Kathleen Moore, Jasgit Sachdev, Monica Mita, Shaveta Vinayak, Shelly Seward, Vassiliki Karantza, Gursel Aktan, Andrew Ferguson, Dmitri Bobilev, Ursula Matulonis. Phase 1/2 study of niraparib plus pembrolizumab in patients with triple-negative breast cancer or recurrent ovarian cancer (KEYNOTE-162). (2016) Abstract TPS5599, 2016 ASCO Meeting, Chicago, IL.

Presentations in National Meetings:

- | | |
|------|---|
| 2010 | A gene expression profile of BRCAness that correlates with responsiveness to platinum, PARP inhibitors and with outcome in epithelial ovarian cancer / Annual American Society of Clinical Oncology (ASCO) Meeting, Chicago, IL |
| 2011 | Development of a Gene Expression Profile of BRCAness that Correlates with Responsiveness to Chemotherapy and Outcome in Epithelial Ovarian Cancer / National Cancer Institute (NCI) Translational Science Meeting, Washington, DC |
| 2012 | Association of a BRCAness profile with outcome and molecular aberrations involving homologous recombination in The Cancer Genome Atlas (TCGA) ovarian cancer dataset / Ovarian Cancer: Molecular Mechanisms and Personalized Medicine. University of Pittsburgh Medical Center (UPMC), Pittsburgh, PA |
| 2013 | Genomic approaches to enhance sensitivity to double-strand DNA break-inducing agents in epithelial ovarian cancer. Department of Defense (DOD) Ovarian Cancer Academy Meeting, Boston, MA |
| 2015 | A Unique Subset of Epithelial Ovarian Cancers with Platinum Sensitivity and PARP-Inhibitor Resistance / Society of Gynecologic Oncology (SGO) Meeting, Chicago, IL |
| 2016 | Selection strategies for immunotherapy for patients with gynecologic malignancies / Society of Gynecologic Oncology (SGO) Meeting 2016, San Diego, CA |

- 2016 Extending PARP-inhibitors in HR proficient ovarian cancers / Stand Up To Cancer (SU2C) Scientific Session: Genomics and beyond - bringing personalized medicine to cancer therapy, American Association of Cancer Research (AACR) Meeting, New Orleans, LA
- 2016 Evaluating novel combinations for synthetic lethality / Combined GYN Developmental Therapeutics/Phase I/Translational Science Workshop. NSABP / RTOG / GOG (NRG) Meeting, Dallas, TX

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

The PI who has worked on this project during the entire duration of the award is Panagiotis Konstantinopoulos.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

We here include the references relevant to this report.

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